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Low-Phosphate Processed Cheese Diminishes Diclofenac-Induced Hepato-Renal Injury in Male Rats

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ABSTRACT

Traditional emulsifying salts used in processed cheesemaking contain high concentrations of sodium and phosphorus, which may represent a health threat to some individuals, especially with chronic kidney and liver diseases. Therefore, it is urgent to search for safe alternatives to commercial phosphate-containing emulsifiers to be used in the manufacture of low-phosphorus processed cheese spread (LP-PCS). Thereby, the present study was undertaken to evaluate the effect of administrating LP-PCS on renal and liver functions and kidney histopathological examination in diclofenac (DF)-treated rats. Consequently, 4 LP-PCS cheeses were manufactured using plant polysaccharides (PP), milk fat globule membrane (MFGM), milk protein concentrate (MPC), or sodium citrate (CIT) as alternatives to commercial emulsifiers. Cheeses coded as PP-, MFGM-, MPC- and CIT-cheese. Besides, cheese with commercial emulsifier was manufactured and served as control (CONT). The experiment lasted for 8 weeks. Results revealed that DF treatment led to significant damage in liver and kidney functions. Both PP- and MFGM- cheeses appeared to have a protective effect against the side effects induced by DF treatment. The protective effect was evident as animals administrated such cheese had biochemical parameters and renal histopathological structure similar to those of healthy animals. In conclusion, the results presented in this study indicated promising protective effects of PP- and MFGM- cheeses against hepatic and renal damages induced by diclofenac administration in rats. Thus, it could be recommended to use PP or MFGM as an efficient replacer to commercial emulsifiers usually used in the production of processed cheese spread.

Key words: diclofenac, emulsifying salts, liver, kidney, processed cheese

INTRODUCTION

Processed cheese and its analogs are considered to be a good source of proteins, fats, minerals, and vitamins. It is usually manufactured by mixing ingredients (mainly natural cheeses, butter, stabilizers, emulsifiers, and others) at temperatures that ordinarily extend between 80 and 100°C under a partial vacuum (Lu *et al.*, 2007).

The consumption of processed cheese and its derivatives grows steadily worldwide and gains significant acceptance particularly among infants and children. However, the increase in global consumption of processed cheese has been associated with growth in demand for a product with improved nutritional quality by reducing concentrations of certain components (e.g., fat, sodium, phosphorus, etc..) or increasing others "protein, Fe, Ca, etc…" (Guinee & O'Kennedy, 2012)

During the production of processed cheeses, emulsifying salts play a critical role and ensure that homogeneous products with craved consistency are made. The real impact of emulsifying salts lies within the exchange of calcium cations of insoluble calcium paracaseinate for sodium particles, which leads to the formation of more dissolvable sodium paracaseinate (Mizuno & Lucey, 2007). The application of emulsifying salts comes about in chain poetization, scattering, hydration, and swelling of proteins, and emulsification and stabilization of fat (Guinee *et al.*, 2004). Emulsifying salts have a significant effect on pH, texture, microstructure, meltability, and sensory attributes as well as functional

properties of processed cheese (Glenn Iii *et al.*, 2003; Acharya & Mistry, 2005). To date, the majority of emulsifying salts used for the manufacture of processed cheese are belonging to phosphate-based salts.

Traditional emulsifying salts used in processed cheesemaking contain high concentrations of sodium and phosphorus. These minerals have important roles in human metabolism when administrated at the recommended daily intake concentration. However, the excessive consumption of both elements may have an adverse effect and cause health problems. The consumption of a high amount of sodium in human nutrition is considered a risk factor for numerous diseases (Palar & Sturm, 2009), mainly hypertension and cardiovascular disease (He & MacGregor, 2007). On the other hand, the excessive consumption of phosphorus or kidney dysfunction has been linked to hyperphosphatemia (Nerbass *et al.*, 2019). The term "hyperphosphatemia" refers to the occurrences of elevated concentration of phosphate in the blood which may lead to the development of calcium deposits in the soft tissue. Hyperphosphatemia is usually associated with complications of bone mineral metabolism and mortality and can be controlled by phosphate binder-based drugs or renal dialysis (Wald *et al.*, 2008).

To avoid health problems linked to excessive consumption of foods containing high levels of sodium and phosphorus, attempts have been made to develop processed cheese with low levels of both minerals by replacement, either partially or completely, of traditional phosphate emulsifying salts with other convenient compounds such as polysaccharide- and protein-based hydrocolloids (Schäffer *et al.*, 1999; Schäffer *et al.*, 2001). The most commonly used polysaccharide-based hydrocolloids are natural and modified starches, carrageenan, pectin, xanthan, and beetle bean gum. However, gelatine, casein, caseinates, and whey proteins are commonly used in processed cheese formulation as protein-based hydrocolloids (Dickinson, 2003; Tan *et al.*, 2007). Recently, Abdo (2021) attempted to replace partially phosphate-based emulsifier with plant polysaccharides from Jew's-mallow (*Corchorus olitorius*), milk fat globule membrane (MFGM), milk protein concentrate (MPC), or sodium citrate in the manufacture of processed cheese spread. Results indicate that up to 60% of emulsifier salts could successfully be replaced by plant polysaccharides without impairing cheese characteristics. However, emulsifiers could totally be replaced by milk fat globule membrane or sodium citrate. Also, MPC could successfully replace 80% of commercial phosphate. In this study, we aimed to evaluate the effect of administrating such cheese on the renal and liver functions (assessed by a panel of laboratory parameters in serum) and kidney histopathological structure in rats suffering from renal chronic disease.

MATERIALS AND METHODS

Processed cheese spread production

Ingredients

Ingredients used for the manufacture of processed cheese were as follows:

Butter (18% moisture and 82% fat), skim milk powder (SMP, 33.4% protein) were obtained from Fonterra Inc. (Auckland, New Zealand), Milk protein concentrate (MPC, 70% protein from Anbros Inc., Singapore), emulsifying salts (polyphosphate Type S2 from Nantong Alchemy Biotech, Jiangsu, China), Cream (30% fat) from Juhayna food industries 6th of October, Giza, Egypt), monoglycerides 95% (from KDVIN food Co. China), Carrageenan (from Araneta Avenue, Quezon City, Philippines), nisin (from Proguiga Biotech, Locurna, Spain) and sodium citrate (PH 8.5) (from Nantong alchemy Biotech, Jiangsu, China),

Milk fat globule membrane (MFGM)was prepared from sweet buttermilk as recently described by ElSaadany & Abd-Elhaleem (2019).

Plant polysaccharides were extracted from stems of Jew's-mallow (*Corchorus olitorius*) with 0.2M lactic acid according to the method described recently by Dabour *et al.* (2019).

Cheese manufacture

Five blends of phosphate-reduced spread processed cheeses were prepared according to the formulations presented in Table (1). Blends were manufactured at the Dairy pilot plant at Dairy Science and Technology Department, Alexandria University. A blend of 2 kg of each cheese trial was prepared in triplicate. Processing was carried out at 85-90ºC with continuous stirring at 1500 rpm for 6-7 minutes using a 2 kg capacity batch Thermomix™ Tm5 (Thermomix, Overwork Corporate Group, which was invented by Cark Vorwerk, France) as described by Kosikowski (1982). Following processing, the melted mixture was poured into glass sterilized jars and kept at 4ºC to serve the animals feeding experiment.

Table 1: Formulation and codes of experimental cheeses were prepared in this study

Ingredients (Kg)	Cheese codes				
	CONT	PP	MFGM	MPC	CIT
Cream 40% fat	32.0	32.00	32.00	32.00	32.00
Butter	16.0	16.00	16.00	16.00	16.00
Skim milk powder	10.0	10.00	10.00	10.00	10.00
Milk protein concentrate	5.00	5.00	5.00	6.06	5.00
Emulsifying salts	1.50	0.60	0.00	0.30	0.30
Citric acid	0.02	0.02	0.02	0.02	0.02
Salt	0.30	0.30	0.30	0.30	0.30
Polysaccharides	0.00	0.76	0.00	0.00	0.00
MFGM	0.00	0.00	1.36	0.00	0.00
Sodium citrate	0.00	0.00	0.00	0.00	1.36
Carrageenan	0.00	0.04	0.04	0.04	0.04
Monoglycerides90%	0.00	0.10	0.10	0.10	0.10
Nisin	0.012	0.012	0.012	0.012	0.012
Potassium sorbet	0.15	0.15	0.15	0.15	0.15
Water	35.00	35.00	35.00	35.00	35.00
Total	100	100	100	100	100
Reduction of emulsifying salts (%)	0	60	100	80	100

The manufactured cheeses were
CONT-cheese: standard cheese formulated with 1.5% commercial emulsifier salts
PP-cheese: cheese in which 60% of commercial emulsifier salts were replaced with polysaccharides extracted from Jew's-mallow stems,
MFGM-cheese: cheese in which 100% of commercial emulsifier salts were replaced with milk fat globule membraned extracted from sweet buttermilk
MPC-cheese: cheese in which 80% of commercial emulsifier salts were replaced with milk protein concentrate, and
CIT-cheese: cheese in which 100% of commercial emulsifier salts were replaced with sodium citrate.

Cheese gross chemical composition and pH

Twenty-four hours following manufacture, cheeses were analyzed for moisture, fat, and salt contents according to the Association of Official Analytical Chemists [AOAC] (1995). Total protein was determined according to the method described by the International Dairy Federation [IDF] (1993) using Kjeldahl Semi-automized Foss model 8100 Dairy Analyzer (FOSS analytical, Hilleroed, Denmark). The pH was measured in cheese slurry, prepared by macerating 20 g of cheese in 20 ml of distilled water, using pH meter (Crison instrument, Spain) as described by Aly *et al.* (2017).

Inductively Coupled Plasma Optical Emission Spectrometer (ICP–OES)

Briefly, 5 g of each cheese and experimental diet was ashed in a porcelain crucible and solubilized with 10 mL of 6 N HCl, then diluted with double-deionized water and filtered with ashless filter paper (Whatman 42, International Ltd., Maidstone, England). The final volume was adjusted to 50 mL. The concentrations of Ca, K, Mg, Na, and P were measured with ICP–OES spectrometer (Varian Vista-Pro, Mulgrave, Victoria, Australia) according to the method proposed by (Temiz & Soylu, 2012). The argon gas used was of spectral purity (99.998%). The plasma gas flow was 15 mL/min, the auxiliary gas flow was 1.5 mL/min and the sample flow rate was 1.5 mL/min. For each of the minerals in the yogurt samples, calibration curves were drawn with five different concentrations of standard solution at a wavelength given in the software of the ICP-OES and were appropriate for the analysis of the device. These curves were plotted according to the concentrations calculated by the device against five different concentrations of the standard solution. For each metal, the reading was carried out against the blind at the specified wavelength. All the analyses were replicated twice.

In vivo experiment

Animals

Seventy adult male albino rats (weighing 140 - 170 g) were housed in the animal house of the Institute of Graduate Studies and Research (IGSR), Alexandria University. The experiment was approved by the local ethical guidelines of the Institutional Animal Care and Use Committee (IACUC), Alexandria University, Egypt (AU08190212209), and all the methods were performed according to regulations of the same committee. Animals were housed in plastic cages (10 rats/cage), with free access to water and a standard diet formulated according to Reeves et al. (1993) as shown in Table (2). Animals were kept under the proper environmental condition at 25±1°C and room humidity of 50±5% with 12 h dark and light on cycle.

Table 2: Formulation of experimental diet

Diet ingredients	Percentage (%)
Whey protein concentrate (80%)	20
Corn starch	50
Corn oil	10
Sucrose	10
Cellulose	5.5
Mineral mixture	3.5
Vitamin mixture	1
The concentration of certain minerals in the formulated diet (µg/g) *1	
Ca	654.8±10.0
K	31.1±2.0
Mg	20.6±0.9
Na	53.7±1.9
P	33.0±1.4

*1Determined by Inductively Coupled Plasma Optical Emission Spectrometer (ICP–OES).

Treatment and protocol

Diclofenac sodium ampoules each one contains 75 mg diclofenac produced by Tabuk Pharmaceutical Mfg. CO. Tabuk Saudi Arabia was obtained from a local pharmacy. Animals were subjected to the following protocol

- *Week 1: intra-gastric administration* of diclofenac at 5 mg/kg body weight (BW)/day,
- Weeks 2-3: *intra-gastric* administration of diclofenac at 2.5 mg/kg BW/day, and
- Weeks 4-8: day-after-day *intra-gastric* administration of diclofenac at 1.25 mg/kg BW.

Experimental design

Animals were acclimatized for 2 weeks and randomly assigned to seven experimental groups of 10 rats each and were subjected to the following groups:

G-1: Untreated animals used as a control group,

G-2: Animals administrated 2 g CONT-cheese/day by gavage,

G-3: Animals administrated 2 g PP-cheese/day by gavage,

G-4: Animals administrated 2 g MFGM-cheese/day by gavage,

G-5: Animals administrated 2 g MPC-cheese/day by gavage,

G-6: Animals administrated 2 g CIT-cheese/day by gavage, and

G-7: Animals administrated a solution of phosphate salts (0.003g/day) by gavage.

The phosphate salt was used for the manufacture of CONT-cheese. It was dissolved in 2 ml distilled water. The administrated volume contained a similar amount of phosphorus equivalent to that of 2 g of control cheese.

Collection of blood samples and tissues

The experiment lasted for 8 weeks. Blood samples of different experimental groups were collected just before starting diclofenac administration (baseline) and after 15 days of cheese administration to ensure the induction and persistence of chronic kidney disease. The collected samples were subjected to measurement of creatinine and urea**.** The baseline levels of serum urea in control and DF-treated animals were 22 and 46 mg/dL, respectively. While serum concentrations of creatinine were 0.4 and 1.1 mg/dL for the same animal groups at the baseline stage and after 15 days of CONT-cheese administration. At the end of the experiment, animals were fasted overnight and lightly anesthetized with isoflurane. Rats were sacrificed and organs (liver, kidney, lung, heart, and testes) were immediately removed, washed twice with saline solution, dried using filter paper, weighed, and stored at -80°C until used. Before freezing, fresh kidneys were subjected directly to histological analysis as mentioned below. Blood samples were centrifuged at 1500 rpm for 20 min to separate sera which were subjected to different biochemical assays. Kidneys were collected via dissection and washed in an ice-cold 1.15%

KCl solution. The kidneys were homogenized with 0.1M phosphate buffer (pH 7.2) and centrifuged at 1500 rpm for 20 min. The supernatant was decanted and evaluated for oxidative stress indices and biochemical parameters.

Body and organs weight

Gain weight of each animal was determined weekly and results were expressed as total weight gained through the entire experiment as described by Elsaadany and Abd-Elhaleem (2019) using the following equation: Gain weight = W_f - W_0.

Where W_f and W_0 were final and initial weights recorded at the end and the beginning of the experiment.

Biochemical assays

Serum calcium and phosphorus concentrations were determined by the methods described previously (Harirforoosh & Jamali, 2005). Total contents of serum proteins and lipids were determined according to the methods described by Lowry *et al.* (1951) and Frings *et al.* (1972), respectively. Serum concentrations of urea and creatinine were determined according to the methods previously described by Uchiyama *et al.* (2016) using commercial kits obtained from Biodiagnostic Company (Dokky, Giza, Egypt). Serum alkaline phosphatase (ALP) activity was assayed by a kinetic method using commercial kits obtained from Spectrum Company (October city industrial area, Cairo, Egypt) according to the method of the International Federation of Clinical Chemistry (Tietz *et al.*, 1983). The activities of superoxide dismutase and glutathione peroxidase and total bilirubin in serum were assayed by the method described by McCord & Fridovich (1969), Rotruck *et al.* (1973), and Rutkowski & Debaare (1966). respectively, using commercial kits (Biodiagnostic Company).

Histopathological examination

Kidney histopathological examination was carried out according to the method of Bancroft *et al.* (1996). Kidney tissue samples were fixed in 10% formalin solution for 18 h and dehydrated through ascending grades of ethyl alcohol. Tissues were then soaked in xylol for 10 min and immersed in molted paraffin wax (56°C). For sectioning, the paraffin blocks were mounted in a microtome. Sections were collected on glass slides and fixed to glass by exposure to indirect heating. Slides were immersed in xylol for three min to dissolve the paraffin and then transferred to absolute alcohol for one min to remove the xylitol. Then sections were dehydrated using a serial solution of ethanol from 95 down to 50%. For staining, sections were dipped in hematoxylin for ten min, washed with distilled water. The excess stain was removed with distilled water and sections were dehydrated by passing through a series of 70, 80, 90, and 95% alcohol for 2 min each, then twice in 100% alcohol. Sections were embedded in Canada balsam, covered with a thin cover glass, and then dried in an oven (40°C) to harden the balsam. Sections were subjected to histopathological examination.

Statistical analysis

Experimental data were statistically analyzed with one-way ANOVA and multiple range tests and expressed as mean values ± SE. Effects with a probability of $P < 0.05$ were considered significant. Statistical analyses were performed using SPSS for windows (Standard Version 16 SPSS Inc. Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Cheese composition

Results concerning the gross composition of experimental processed cheeses are shown in Table 3. The pH values of different processed cheeses ranged from 5.5 to 5.7and no significant differences ($P \geq 0.05$) were observed among cheeses. The ability of emulsifying agents used in the present study to maintain pH at the range of 5.5 to 5.7 seems to be important to improve the configuration and solubility of casein leading to the formation of processed cheese with a homogenous structure (Marchesseau *et al.*, 1997). The results also indicated a significant increase ($P \leq 0.05$) in fat and protein contents in MFGM- and MPC-cheeses compared with CONT-cheese. Both MFGM and MPC might bring additional fat and protein to the final cheese. However, there were no significant differences in fat and protein contents among cheeses subjected to treatments PP or CIT compared with control cheese (CONT). The dry matter content in the processed cheese was affected by the substitution of emulsifying salts, where the dry matter content increased significantly in PP-, MFGM-, MPC, and CIT-cheese compared with CONT. Salt content did not differ among cheeses ($P \geq 0.05$) has ranged from 0.55% to 0.60%. All experimental cheeses made in the present study were formulated to full-fat the require-

ments of the Egyptian standard specification for full-fat processed cheese spreads (35-65% fat/dry matter and at least 44% dry matter) (Egyptian standards No 999, 2013).

The contents of some minerals (mainly Ca, K Mg, Na, and P) in different cheeses are presented in Table (3). It was obvious that the substitution of commercial emulsifying salts had a significant effect on the mineral content of processed cheese. All cheeses had a significantly lower concentration of P compared with CONT-cheese. The content of P was reduced by 27, 57, 61, and 50% in PP-, MFGM-, MPC- and CIT-, respectively. The replacement with PP and MFGM led to a significant increase in the content of Ca, Mg, and K and a reduction in Na and P in the final cheese compared with CONT-cheese. Among cheeses, MPC-cheese had the lowest content of Ca, Mg, K, and Na. CIT-cheese had a significantly higher content of Ca, Mg, K, and Na compared with CONT-cheese.

Diclofenac and kidney function

Diclofenac, a nonsteroidal anti-inflammatory drug, has many adverse effects on renal function. It is safe at therapeutic doses but induces toxicity in humans and animals when administered in large doses (Owumi & Dim, 2019). The toxicity of DF is associated with its reactive metabolites, including 4-OH-DCF and 5-OH-DCF, and the highly reactive benzoquinone imines (Boerma *et al.*, 2012; Lazarska *et al.*, 2018). It reduces the synthesis of renal prostaglandins (Bosch-Marcè *et al.*, 1999), induces oxidative stress, and causes nephrotoxicity and acute kidney injury (Alkuraishy *et al.*, 2019). In this study, blood samples collected from DF-treated animals, just before starting administration of cheeses, indicated that one week of oral administration of diclofenac (5 mg/kg BW/day/animal) resulted in a significant elevation of urea and creatinine (Table 4). The levels of urea and creatinine in blood samples of normal animals increased from 22 and 0.40 mg/dl, respectively, to 46 and 1.1 mg/dl in blood samples collected from DF-treated animals. In general, the presence of high levels of urea in urine or blood, known medically as uremia, has been usually taken as an indicator of kidney damage. Alkuraishy *et al.* (2019) reported that administration of diclofenac led to a significant increase in blood urea and serum creatinine. Sivaraj & Umarani (2018) evaluated the effect of different doses of diclofenac (0, 10, 50, and 100 mg/kg body weight) on renal function in the rat. in a concentration-dependent manner, results indicated that diclofenac treatment reduced urinary output, renal plasma flow, and the glomerular filtration rate.

Fifteen days following cheese administration, blood samples of each animal group were collected and subjected to measurement of urea and creati-

Table 3: Gross chemical composition, pH, and concentration of some minerals of processed cheese spreads in which commercial phosphate–based emulsifier was replaced with vegetable polysaccharides, milk fat globule membrane, milk protein concentrate or sodium citrate. Data are the means ± standard error.

Cheese codes*[1]	pH	Fat (%)	DM (%)	Fat/dry matter[2]	Salt (%)	Protein (%)	Minerals (µg/g)				
							Ca	K	Mg	Na	P
CONT	5.7±0.01^{a}	25.7±0.3^{b}	44.6±0.5^{c}	57.5±1.3^{b}	0.55±0.01^{a}	7.5±0.7^{c}	112.6±6.0^{c}	95.0±5.0^{c}	9.8±0.4^{c}	125.3±4.3^{b}	219.6±12.0^{a}
PP	5.6±0.0^{a}	25.5±0.5^{b}	42.9±0.1^{d}	59.4±1.2^{a}	0.58±0.0^{a}	7.69±0.1^{c}	123.0±7.0^{b}	124.1±9.0^{a}	13.2±0.9^{a}	101.2±8.0^{c}	161.7±10.0^{b}
MFGM	5.7±0.0^{a}	26.0±0.5$^{a.b}$	43.3±0.3^{b}	59.3±1.0^{a}	0.59±0.0^{a}	9.04±0.0^{b}	128.4±9.3^{b}	97.9±11.5^{b}	11.7±0.5^{b}	47.4±3.3^{d}	95.4±6.6^{d}
MPC	5.6±0.0^{a}	26.2±0.3a	42.9±0.1^{c}	60.9±0.7^{a}	0.60±0.0^{a}	11.30±0.0^{a}	91.2±5.6^{d}	69.2±3.2^{d}	5.8±0.8^{d}	46.8±1.0^{d}	86.3±5.2^{e}
CIT	5.5±0.01^{a}	25.5±0.5^{b}	43.1±0.2^{d}	59.1±0.9^{a}	0.59±0.0^{a}	7.76±0.0^{c}	158.9±7.8^{a}	97.9±7.0^{b}	12.9±0.9^{a}	136.6±9.8^{a}	111.1±7.5^{c}

*[1]CONT-cheese: standard cheese formulated with 1.5% emulsifier commercial salts,

PP-cheese: cheese in which 60% of commercial emulsifier salts were replaced with stems,

MFGM-cheese: cheese in which 100% of commercial emulsifier salts were replaced with milk fat globule membraned extracted from sweet buttermilk

MPC-cheese: cheese in which 80% of commercial emulsifier salts were replaced with milk protein concentrate, and

CIT-cheese: cheese in which 100% of commercial emulsifier salts were replaced with sodium citrate.

Superscript letters in the same column denote significant differences ($P \leq 0.05$).

Table 4: Levels of urea and creatinine (mg/dL) in blood and the number of dead animals during the experiment. Data are the means ± standard error.

Stage of experiment/Cheese codes*1	Urea	Creatinine	Number of deaths (of 10 animals)*2
The baseline for animals subjected to G-1*3	22.00±0.30^{f}	0.40±01^{f}	-
The baseline for animals subjected to G-2 to G-7*3	46.00±0.20^{c}	1.10±0.5^{b}	-
Fifteen days following administration of experimental cheeses			
G-1	25.00±0.30^{e}	0.50±0.00^{e}	0
G-2	68.00±1.89^{b}	1.60±0.89^{a}	4
G-3	39.00±2.56^{d}	1.04±0.03^{c}	1
G-4	37.00±2.90^{d}	0.92±0.09^{d}	2
G-5	42.00±1.05^{c}	1.09±0.12^{b,c}	2
G-6	45.00±5.11^{c}	1.09±0.87^{b}	5
G-7	74.00±5.45^{a}	1.70±0.56^{a}	4

*1 **G-1**: Untreated healthy animals, **G-2:** RCD-induced animals administrated 2 g CONT-cheese/day by gavage, **G-3**: RCD-induced animals administrated 2 g PP-cheese/day by gavage, **G-4**: RCD-induced animals administrated 2 g MFGM-cheese/day by gavage, **G-5**: RCD-induced animals administrated 2 g MPC-cheese/day by gavage, **G-6**: RCD-induced animals administrated 2 g CIT-cheese/day by gavage, and **G-7**: RCD-induced animals administrated solution of phosphate salts (0.003g. /day) by gavage. The phosphate salt was powder used for the formulation of control was dissolved in 2 ml water and the administrated volume contained a similar amount of phosphorus equivalent to that of 2 g control cheese.

Superscript letters in the same column denote significant differences ($P \leq 0.05$).

*2 Recorded at the end of the experiment (after 52 days).

*3 Analysis were carried out at the end of week 1 (just before starting administration of cheeses) through which animals were subjected to a daily intragastric administration of diclofenac at a concentration of 5 mg/kg body weight (BW)/day/animal.

nine. This was to evaluate the effect of short-term administration of processed cheese made with emulsifying salt replacers on kidney function in DF-t rats. The concentrations of urea of different animal groups were in the order G-7> G-2> G-6> G-5> G-3> G-4> G-1, respectively. The levels of blood creatinine were in the order G-7> G-2> G-6> - G-5> G-3> G-4> G-1, respectively.

The establishment of kidney failure in animals was accompanied by remarkably visible symptoms. DF-t animals (G-2 to G-7) became less active and had a faster breathing rate compared with healthy ones (G-1). Symptoms of kidney disorder were evident among DF-t animals as their ears, claws, tails, eyes, and noses became pale, and the fur was sparse and rough. Also, the fur between the thighs became wet and yellowish in color, indicative of frequent urination. These characters became worst in animals subjected to treatment G7 and improved in animals belonged to G-3 and G-4.

The incidence of mortality among experimental groups is presented in Table (4). The mortality rate among DF-t animals appeared to correlate with the type of emulsifying salts replacer added to the cheese. Animals fed processed cheese with sodium citrate (G-5) had the highest rate of mortality (5 deaths/10 animals) while those fed cheese made with plant polysaccharides (G-3) showed the least rate (1 death/10 animals). However, no deaths were recorded for the control group (G-1). On the other hand, the numbers of deaths for groups G-7 and G-2 were 4 animals for each group while 2 animals died in groups G-4 and G-5. The number of deaths reported in this study might refer to the development of severe complications due to diclofenac treatment.

The high rate of mortality in animals subjected to G-6 might be attributed to sodium citrate used to emulsify CIT-cheese and probably due to the formation of stone in the kidney and/or the increment in blood pressure. Although, alkali therapy (treatment with sodium or potassium citrates) is medically used to prevent stone formation in patients with kidney disease. Sodium citrate, contrary to potassium citrate, has been shown to induce the crystal-

lization of calcium salts in patients with distal renal tubular acidosis(Sakhaee *et al.*, 1983; Preminger *et al.*, 1988). On the other hand, a healthy kidney is known to be efficient at regulating sodium content in the human body and thus controlling blood volume and pressure (Ellison, 2017). In the present study and among tested cheeses, the serum of DF-treated animals that administrated CIT-cheese (G-6) showed the highest sodium concentration even higher than those received control cheese (G-2) (as discussed below). High sodium content is known to increase blood hypertension (Ellison, 2017).

The lowest rate of mortality reported in this study for animals belonged to G-3 might indicate that polysaccharides extracted from Jew's-mallow had the most protective effect against complications associated with RCD. Indeed, some plant polymers have been reported to improve kidney function in patients with kidney problems. For example, gum acacia was found to be a useful dietary agent in attenuating the progression of chronic kidney disease in humans Elamin *et al.* (2017) and rats Al Za'abi *et al.* (2018). The mechanisms by which plant polymers can abate the RCD may be attributed to their ability to reduce the levels of inflammatory cytokines and oxidative and nitrosative stress markers (Al Za'abi *et al.*, 2018). Polysaccharides of Jew's-mallow might act similarly.

Body gain and relative organ weights

In general, diclofenac treatment resulted in a significant decrease in the absolute body weight of rats (Data have not shown). Body gain and organs relative weights of different animal groups as determined at the end of the experiment (Table 5). The body gain weight of DF treated animals were significantly ($P < 0.05$) lower compared with normal rats (G-1). Similarly,

Al Za'abi *et al.* (2018) and Owumi & Dim (2019) reported that the induction of RCD in rats by DF administration resulted in a significant reduction in body weight gain compared with untreated rats. On the other hand, animals administrated processed cheeses (G-2 to G-6) responded differently to DF administration depending on the type of cheese. Contrary to animals subjected to G-2, significant ($P < 0.05$) increases in body gain weight were noted in animals belonged to G-3, G-4, and G-5 while those in G-7 had the least ($P > 0.05$) gained weight. Animals administrated CIT-cheese (G-6) had gain weight lower ($P > 0.05$) compared with that of G-2.

Compared with the normal control group (G-1) DF treated groups (G-2-G-7) had a significant ($P < 0.05$) increase in the relative weight of different organs (Kidney, Liver, heart, Testicles, and spleen) depending on the type of administrated cheeses. Al Za'abi *et al.* (2018) and Owumi & Dim (2019) also reported that animals treated with DF had kidney and liver relative weights significantly higher than those for untreated rats. Also, Sallie *et al.* (1991) reported that DF treatment led to renal and hepatomegaly as a response to its hepatotoxic effect. In this study animals administrated PP- or MFGM-cheeses appeared to have organs relative weight better than those received cheese with sodium citrate or MPC. This may indicate the beneficial effect of Jew's-mallow polysaccharides and MFGM to reduce the complications associated with DF treatment.

Table 5: Body gain and organs relative weights of control and diclofenac-treated rats administrated different processed cheese spreads. Data are expressed as mean values ± standard error.

Animal groups*1	Body gain weight (g)	Relative organs weight (%)				
		Liver	Kidney	Heart	Testicles	Spleen
G-1	60.5±1.22^{a}	3.57±0.33^{f}	0.85±0.05^{e}	0.42±0.02^{b}	1.40±0.15^{c}	0.83±0.05^{b}
G-2	14.6±1.2^{e}	5.18±0.61^{a}	1.16±0.08^{d}	0.55±0.05^{a}	2.04±0.26^{a,b}	1.04±0.17^{a}
G-3	22.7±2.2^{c}	4.40±0.70^{e}	1.18±0.25^{d}	0.53±0.05^{a}	1.81±0.04^{b}	1.05±0.20^{a}
G-4	25.0±2.1^{b}	4.58±0.47^{d,e}	1.20±0.23^{d}	0.51±0.06^{a}	1.83±0.37^{b}	1.06±0.13^{a}
G-5	18.2±0.2^{d}	5.03±0.79^{a,b}	1.42±0.16^{b}	0.50±0.04^{a}	1.96±0.33^{b}	1.03±0.16^{a}
G-6	14.2±1.2^{e}	4.87±0.78^{b,c}	1.56±0.20^{a}	0.52±0.01^{a}	2.34±0.41^{a}	1.04±0.12^{a}
G-7	11.00±1.1^{f}	4.78±0.25^{c,d}	1.32±0.08^{c}	0.52±0.02^{a}	2.01±0.12^{a,b}	1.04±0.04^{a}

*1 See footnote Table 4

Superscript letters in the same column denote significant differences ($P \leq 0.05$).

Kidney function

Data in Table (6) show the levels of renal function markers (urea and creatinine) in serum samples analyzed at the end of the experiment. The chronic renal damage induced by DF in rats, as evidenced by the significant elevation of serum urea and creatinine, continued during the entire experimental period. This harmful defect was remarkable particularly in animals subjected to treatments G-2 and G-7. Similarly, Mostafa *et al.* (2020) reported that the administration of diclofenac (50-300 mg/kg) could result in elevation of serum creatinine and urea up to 272% and 217% respectively as compared to the normal control group. The elevation of creatinine and urea in the blood of DF-T animals indicated that diclofenac compromised the integrity of the glomerular filtration rate barrier, leading to impaired renal function (Farombi *et al.*, 2002; Maalej *et al.*, 2017). On the other hand, animals administrated processed cheeses with the selected concentrations of replacers of commercial emulsifier had variable amelioration rates of renal function depending on the type of added material. Cheese with MFGM (G-4) had the highest rate to restore kidney function as it showed the least ($P \leq 0.05$) concentrations of urea and creatinine among DF-T cheeses, followed by cheeses with plant polysaccharides (G-3), MPC (G-5) and sodium citrate (G-6), respectively. Animals subjected to G-2 and G-7 had significant ($P < 0.05$) increases in plasma urea and creatinine levels compared with the control group (G-1).

Serum mineral homeostasis

Table (6) shows also the effect of administrating different chesses on the concentration of some minerals (mainly Ca, P, Na, and K) in sera of DF-T rats. These minerals are representing the main core of the serum mineral homeostasis. Diclofenac treatment appeared to disturb serum mineral homeostasis in rats as a consequence of renal dysfunction. Diclofenac-control group (G-2) had a significant increase in P, Na, and K and a lower concentration of Ca compared with the normal control group (G-1). This finding is in accordance with that of Harirforoosh & Jamali (2005) who reported that chronic kidney disease (CKD) could disrupt mineral homeostasis and its main underlying cause is secondary hyperparathyroidism. CKD is usually associated with the presence of high mineral content, particularly P, in serum as a kidney is not able to eliminate phosphate from the blood properly (Uchiyama *et al.*, 2020).

DF-treated animals subjected to groups G-3 and G-4 had mineral homeostasis very close to those subjected to the control group (G-1), indicating the effective role of plant polysaccharides and MFGM in re-storing the mineral equilibrium in animals with CRD to the normal level. On the other hand, MPC- or sodium citrate-containing cheeses had a very limited capacity to re-establish mineral equilibrium, which emphasizes their low ability to improve the kidney damage induced by diclofenac administration.

Hepatic function and antioxidant status

Table (7) shows levels of protein, lipid, bilirubin and some liver enzymatic biomarkers in plasma of DF-treated rats administrated processed cheese spread emulsified with different agents. In comparison with control animals (G-1), the levels of total

Table 6. Levels of urea, creatinine, and minerals (mg/dL) in the serum of control and diclofenac-treated rats administrated different processed cheese spreads. Data are expressed as mean values ± standard error

Animal groups[*1]	Urea	Creatinine	Calcium	Phosphorous	Sodium	Potassium
G-1	28.0±2.8^{f}	0.75±0.07^{d}	10.4±0.01^{a}	3.1±0.2^{e}	141.4±2.7^{e}	4.0±0.4^{f}
G-2	78.5±6.3^{c}	2.18±0.31^{b}	7.9±0.4^{d}	10.6±2.4^{a}	199.4±7.5^{c}	7.3±0.1^{c}
G-3	30.5±4.9^{e}	0.69±0.05^{d,e}	10.1±0.4^{a}	3.5±0.4^{d,e}	143.0±6.4^{e}	4.8±1.2^{e}
G-4	23.4±3.4^{g}	0.62±0.09^{e}	10.5±0.1^{a}	3.6±0.2^{d,e}	135.1±3.0^{f}	4.0±0.2^{f}
G-5	57.2±4.5^{d}	1.50±0.20^{c}	9.0±2.1^{b}	6.6±3.7^{c}	179.0±6.6^{d}	6.1±0.3^{d}
G-6	91.9±7.9^{b}	2.45±0.21^{a}	8.4±0.6^{c}	6.1±1.6^{c}	285.5±9.5^{b}	12.2±0.4^{a}
G-7	114.0±3.3^{a}	2.50±0.10^{a}	7.4±0.3^{d}	8.8±1.1^{b}	316.1±26.7^{a}	10.8±0.9^{b}

[*1] See footnote Table 4

Superscript letters in the same column denote significant differences ($P \leq 0.05$).

Table 7: Levels of total proteins, total lipids, bilirubin, and liver enzymes in serum of control and diclofenac-treated rats administrated different processed cheese spreads. Data are expressed as mean values ± standard error.

Animal groups*1	Total protein (mg/dL)	Total lipids (mg/dL)	Total bilirubin (µmol/L)	Alkaline phosphatase (U/mg protein)	Glutathione peroxidase (U/mg protein)	Superoxide dismutase (U/mg protein)
G-1	4.95±0.07 [a]	530±39.59 [e]	0.59±0.03 [c,d]	59.5±4.9 [f]	10.4±0.1 [a]	24.8±1.8 [a]
G-2	3.80±0.56[d]	753.5±21.9[a]	0.94±0.05 [a]	101.5±4.9[b]	7.3±0.1 [c]	18.3±1.5[c]
G-3	4.75±0.07[a]	565±127.2[c]	0.39±0.03 [e]	70.5±3.5[e]	8.7±0.1 [b]	23.3±1.9[b]
G-4	4.65±0.35[a,b]	543±57.98 [d]	0.40±0.05[e]	54.5±3.5 [g]	8.9±0.2 [b]	23.6±1.1[b]
G-5	4.35±0.49 [b,c]	613.5±21.92[b]	0.52±0.08 [d]	79.0±4.2[d]	7.6±0.5 [c]	18.2±0.6 [c]
G-6	4.05±0.35[c]	757.0±6.36[a]	0.67±0.12 [c]	87.5±6.4[c]	7.5±0.1[c]	18.0±1.2[c]
G-7	3.80±0.14[d]	759.5±30.4[a]	0.83±0.21[b]	107.5±6.4[a]	7.5±0.1 [c]	17.1±2.1[d]

*1 See footnote Table (4).
Superscript letters in the same column denote significant differences ($P \leq 0.05$).

protein in plasma decreased (P <0.05) in DF-treated animals. On contrary, total fat concentration appeared to increase in the plasma of DF-treated rats. In both cases, PP- and MFGM-cheeses appeared to bring the concentration of protein and fat closer to their normal levels.

Also, DF treated animals had significantly (P <0.05) increased plasma level of bilirubin when compared with the untreated group (G-1). Animals administrated CONT-cheese (G-2) had the highest level of total bilirubin followed by those administrated a solution of phosphate salts (G-7). Animals administrated PP- or MFGM-cheese had the lowest (P <0.05) levels in bilirubin, even lower than control animals (G-1), among DF-treated groups. This may indicate the efficiency of PP and MFGM at controlling liver damage induced by DF and restoring liver function. The elevation in the plasma level of bilirubin in DF-treated rats is usually due to an injury to the biliary duct of the liver (Owumi & Dim, 2019). In accordance with our results, Alabi & Akomolafe (2020) reported increases in total bilirubin and lipids and a reduction in total protein in the plasma of DF-treated rats compared with untreated animals. The authors suggested that these changes might indicate the formation of oxidative stress and inflammation in DF-treated rats.

Alkaline phosphatase (one of the hepatic transaminases) is commonly used as biomarkers for hepatic injury, which leads to the release of the enzyme into the bloodstream (Owumi & Dim, 2019). It is obvious from Table (7) that the administration of DF resulted in significant increases in the activity of ALP in plasma of animals subjected to different groups G-2 to G-7 compared with the control group (G-1). Similarly, previous studies indicated that DF administration caused significant elevation of ALP activity in plasma compared with untreated rats (Peter *et al.*, 2017; González-Ponce *et al.*, 2018; Alabi & Akomolafe, 2020). DF-treated animals administrated the aqueous solution of commercial emulsifying salt (G-7) had the highest ALP activity followed by those of G-2. The activities of ALP were in the following descending order G-6> G-5> G-3> G-1> G-4, respectively. PP- and MFGM-cheeses (G-3 and G-4, respectively) proved again their ability to repair liver damage induced by DF treatment.

Endogenous antioxidant (glutathione peroxidase and superoxide dismutase) have an effective role in the protection of cells from reactive oxygen and nitrogen species, and lipid peroxidation (Owumi & Dim, 2019). In the present study, DF treatment appeared to decrease ($p < 0.05$) both enzymes in plasma (Table 7). This was in accordance with the results reported previously and attributed to the accumulation of toxic metabolites in hepatic and renal tissues due to DF administration (den Braver *et al.*, 2016, Peter *et al.*, 2017). The activities of both enzymes in animals of G-2 and G-7 were the lowest ($p < 0.05$) among DF-treated groups compared with those subjected to control groups (G-1). However, the activities of glutathione peroxidase and superoxide dismutase appeared to improve ($p < 0.05$) in DF-treated animals subjected to groups G-3 and G-4, which may indicate the protective ef-

fect of plant polysaccharides and MFGM against DF-induced oxidative stress. This observation may also confirm the protective effect of both materials against the side effects induced by DF administration, which is in accordance with results of biochemical tests and histopathological examination of both groups reported in this study.

Renal histopathological examination

Kidney sections from normal control (G-1) showed normal glomerulus and tubules with usual morphology. However, histological examination of the kidneys from DF-treated rats (G-2) showed that the histological structure was remarkably different from that of normal animals. The renal corpuscles appeared distorted with hyper-infiltration of the glomerulus. Also, the formation of intensive mesangial matrix and widespread necrosis of the renal tubules could be detected easily. The formation of necrosis of the renal tubules is leading to dilatation of renal vessels, desquamation of tubular cells, the formation of the intraluminal cast, and infiltration of inflammatory leukocytes (Mostafa *et al.*, 2020). Renal sections from animals subjected to group G-7 had typical histopathological order similar to that described to the diclofenac control group (G-2). Our findings were similar to those reported previously by Mostafa *et al.* (2020) for kidneys of normal and DF- treated animals. The authors reported that renal sections from DF-treated rats showed massive areas of fibrosis and ongoing cell apoptosis. However, the glomerular membrane was thicker than normal but tubules were much affected by diclofenac treatment.

Histological analysis of renal sections from DF-treated rats administrated processed cheeses showed less histopathological renal alteration, depending on the type of cheese, compared with those from the G-2 group. Animals that received processed cheeses with MFGM or Jew's-mallow polysaccharides had renal histopathological structures very close to that of animals belonging to the control group (G-1). This may indicate the ability of MFGM and polysaccharides to repair the damage in renal tissues caused by diclofenac. However, cheese with sodium citrate had the least ameliorative effect on renal tissues as kidney sections from G-6 appeared to have histological structure closer to those from G-2.

Renal histopathological evidence of different animal groups, reported in this study, is in accord-

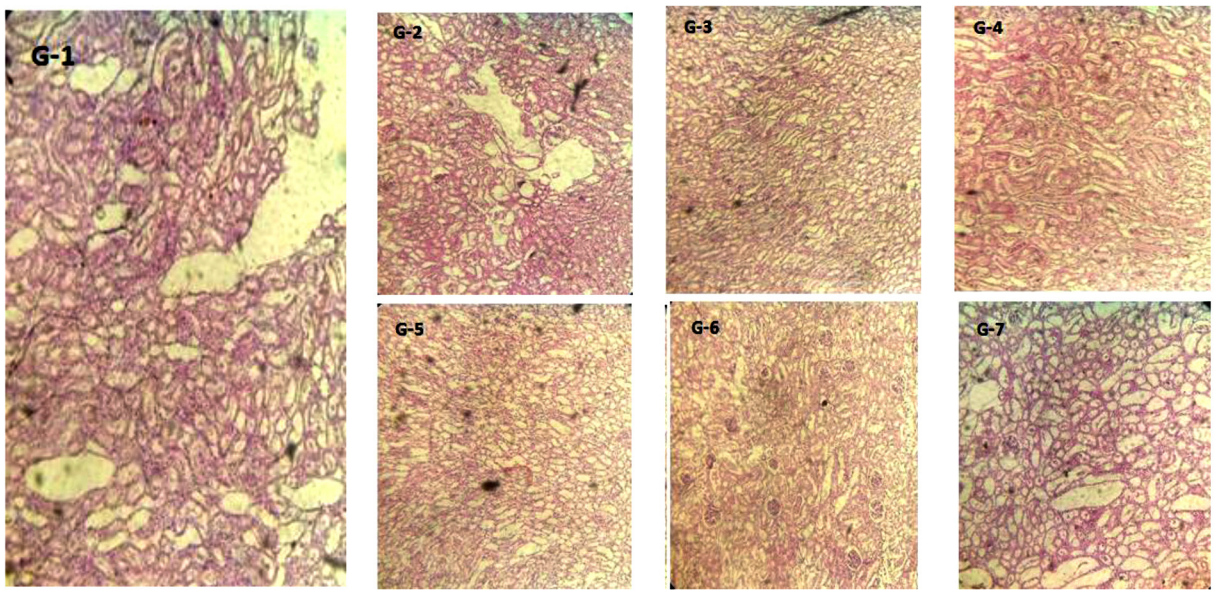

Figure 1: Light photomicrograph of liver sections: G-1: Untreated healthy animals, G-2: RCD-induced animals administrated 2 g CONT-cheese/day by gavage, G-3: RCD-induced animals administrated 2 g PP-cheese/day by gavage, G-4: RCD-induced animals administrated 2 g MFGM-cheese/day by gavage, G-5: RCD-induced animals administrated 2 g MPC-cheese/day by gavage, G-6: RCD-induced animals administrated 2 g CIT-cheese/day by gavage, and G-7: RCD-induced animals administrated solution of phosphate salts (0.003g. /day) by gavage. The phosphate salt was powder used for the formulation of control was dissolved in 2ml. water and the administrated volume contained a similar amount of phosphorus equivalent to that of 2 g control cheese.

ance with the biochemical results and can explain the restoration of kidney functions in animals fed processed cheese allocated to groups G-3 and G-4. Thus, the ability of MFGM and PP to maintain the structural and functional integrity, almost to the same extent as that of the normal control group, of renal tissue in DF-treated animals might also prove the protective effect of these substances to avoid side effects related to diclofenac.

CONCLUSION

This study revealed that MGFM- or polysaccharides-containing processed cheeses could potentially improve the overall status of rats with renal damage induced by diclofenac treatment. The therapeutic impact of these cheeses might come from their ability to restore the normal status for physiological and histopathological functions of rats. Also, such cheese could significantly repair the damage induced by diclofenac via upregulating the antioxidant defenses and restoring renal tissues in animals with kidney failure.

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الجبن المعامل منخفض الفوسفات يقلل من الإصابة الكبدية-الكلوية التي يسببها الديكلوفيناك في ذكور الجرذان

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تحتوي أملاح الاستحلاب التقليدية المستخدمة في صناعة الجبن المعامل على تركيزات عالية من الصوديوم والفوسفور. قد يسبب الاستهلاك المفرط لكلا العنصرين مشاكل صحية. وبالتالي ، فإن الجبن المعامل قد يمثل خطراً صحياً على بعض الأفراد ، وخاصة أولئك الذين يعانون من أمراض الكلى والكبد المزمنة. لذلك ، يجب على الباحثين البحث عن بدائل آمنة للمستحلبات التجارية المحتوية على الفوسفات والتي يمكن استخدامها في تصنيع جبن مطبوخ منخفض فى محتواه من الفوسفور . ومن ثم فقد أجريت هذه الدراسة لتقييم تأثير تناول جبن مطبوخ مصنع ببدائل لأملاح الفوسفات التجارية في ذكور الجرذان المعاملة بالديكلوفيناك لإحداث خلل فى وظائف الكبد و الكلى بالفئران . وبالتالي، تم تصنيع ٤ معاملات من الجبن المعامل باستخدام السكريات النباتية المستخلصة من سيقان نبات الملوخية ، غشاء حبيبات الدهن، مركز بروتين اللبن وسترات الصوديوم. و كانت نسب استبدال أملاح الاستحلاب ٦٠ و ١٠٠ و ٨٠، ١٠٠٪ على الترتيب. كما تم تصنيع عينة من الجبن باستخدام املاح الاستحلاب التجارية ككونترول. واستمرت التجربة لمدة ٨ أسابيع. أوضحت النتائج أن معاملة الجرذان بالديكلوفيناك أدت إلى ارتفاع معنوي في محتوى كل من اليوريا والكرياتينين في مصل الدم ، وانخفاض في أوزان الحيوانات، وزيادة الوزن النسبي للأعضاء واضطراب التوازن المعدني في مصل الدم. و فيما يتعلق بوظائف الكبد وقدرته على إنتاج مضادات الأكسدة الكبدية فقد تأثرت سلبًا بالمعاملة. و أظهر الفحص التشريحي لأنسجة الكلى أن الحيوانات المعاملة بالديكلوفيناك ظهر بها تشوهات فى تركيب الأنسجة مع ارتشاح مفرط فيها. و كان لاستهلاك الجبن المعامل تأثيرات متغيرة على وظائف الكبد والكلى بناءً على المادة المستخدمة لإحلال أملاح الأستحلاب التجارية. بشكل عام ، يبدو أن الجبن المحتوى على السكريات النباتية المستخلصة من سيقان نبات الملوخية أوعلى غشاء حبيبات دهن اللبن لهما تأثير وقائي ضد الآثار الجانبية التي يسببها الديكلوفيناك. و ظهر التأثير الوقائي واضحًا فى الحيوانات التى تناولت أيًا من معاملتى الجبن من خلال القياسات الكيموحيوية وقياسات فحص الأنسجة الكلوية و التى كانت مماثلة لتلك الموجودة في الحيوانات السليمة (المجموعة الضابطة أو الكونترول). ويمكن التوصية باستخدام السكريات النباتية المستخلصة من سيقان نبات الملوخية أوغشاء حبيبات دهن اللبن كبدائل فعالة و صحية لمستحلبات الفوسفات التجارية في إنتاج الجبن المعامل القابل للفرد للأفراد الذين يعانون من مشاكل صحية مع الفوسفور.